Suppression of the SDF-1/CXCR4/β-catenin axis contributes to bladder cancer cell growth inhibition in vitro and in vivo

TAO ZHANG¹, FEI YANG², WENBIAO LI², BOULONG LIU², WENDE LI³, ZHIYI CHEN¹ and CAN WANG¹

¹Department of Urology, Beijing University of Chinese Medicine Shenzhen Hospital, Shenzhen, Guangdong 518172; ²Department of Urology, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510630; ³Guangdong Laboratory Animals Monitoring Institute, Guangzhou, Guangdong 510260, P.R. China

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Abstract. Previous studies have found that the activation of stromal cell-derived factor-1 (SDF-1)/CXCR chemokine receptor-4 (CXCR4)/β-catenin signaling is associated with biological malignant potential in cancers. However, its function has been rarely reported in the progression of bladder cancer (BCa). The aim of the present study was to investigate the association of SDF-1/CXCR4 signaling and β-catenin in regards to BCa cell proliferation, colony formation, migration and invasion. The methods used were MTS, colony formation, and Transwell migration and invasion assays which were performed in SW780 cells following treatment with the CXCR4 antagonist AMD3465, SDF-1, the β-catenin antagonist FH535, AMD3465+SDF-1 or FH535+SDF-1. The mRNA and protein levels were assayed by RT-qPCR and western blotting, respectively. The effect of AMD3465 on SW780 cell xenograft growth in vivo was evaluated using a nude mouse model. According to our results, human BCa SW780 cells were identified as having high expression of CXCR4 and β-catenin. Subsequently, we found that both CXCR4 and β-catenin antagonists could significantly inhibit the proliferation, colony formation, migration and invasion of SW780 cells. Notably, SDF-1 could reverse the inhibitory effects of AMD3465 on proliferation, colony formation, migration and invasion in SW780 cells. In AMD3465-treated SW780 cells, the expression of c-myc was significantly upregulated, and E-cadherin was downregulated in the presence of SDF-1. Furthermore, the tumor volume and average weight in the AMD3465-treated group were evidently less than these parameters in the control group, indicating that AMD3465 can inhibit SW780 cell growth in vivo. In conclusion, targeting the SDF-1/CXCR4/β-catenin axis may be a potential therapeutic target for suppressing BCa progression.

Introduction

Bladder cancer (BCa) is one of the most commonly diagnosed urologic malignant tumor and remains life-threatening due to its high occurrence of metastases. The 5-year BCa survival rate is <60% (1). Each year ~80,500 patients are diagnosed with BCa, and 32,900 cancer-related deaths were reported to a population-based cancer registry (2009-2011) in China (2). Cisplatin-based chemotherapy combined with radical cystectomy is the recommended treatment strategy in routine clinical practice (3,4); however, the outcome of BCa patients is usually ineffective or poorly tolerated. Therefore, it is imperative to explore new potential molecular mechanisms that may provide better therapeutic targets and achieve better therapeutic efficacy to decrease the mortality rate of patients with BCa.

Stromal cell-derived factor-1 (SDF-1) is a member of the cysteine-X-cysteine class of chemokines, and achieves its biological functions by binding to the CXC chemokine receptor 4 (CXCR4) and CXCR7 (5). SDF-1 and CXCR4 are also involved in the progression of BCa (15,16). Gosalbez et al found that the expression of SDF-1 was increased in bladder tumors and was related with high-grade tumors and metastasis (16). CXCR4-positive BCa cells have been associated with poor prognosis in various human cancers, including ovarian (8), head and neck (9), rectal (10) and breast (11) cancers. A growing number of studies has demonstrated that the SDF-1/CXCR4 chemokine pathway promotes the survival, proliferation and migration of cancer cells (12-14), indicating that interruption to the SDF-1/CXCR4 pathway may influence the biology of cancer (7). Upregulation of SDF-1 and CXCR4 has been associated with poor prognosis in various human cancers, including ovarian (8), head and neck (9), rectal (10) and breast (11) cancers. A growing number of studies has demonstrated that the SDF-1/CXCR4 chemokine pathway promotes the survival, proliferation and migration of cancer cells (12-14), indicating that interruption to the SDF-1/CXCR4 axis may be a new therapeutic strategy to inhibit cancer metastases. SDF-1 and CXCR4 are also involved in the progression of BCa (15,16). Gosalbez et al found that the expression of SDF-1 was increased in bladder tumors and was related with high-grade tumors and metastasis (16). CXCR4-positive BCa cell exposure to SDF-1 provoked a significant increase in both cell migration and invasion abilities (17).

Previous studies have demonstrated that SDF-1/CXCR4 may induce cancer cell proliferation, and invasion through activation of the Wnt/β-catenin signaling pathway (18-20). Specifically, the Wnt/β-catenin signaling pathway is a key modulator of cellular proliferation (21). In addition, β-catenin as a transcriptional co-regulator cooperates with transcription
factors to determine gene expression (22). The Wnt/β-catenin signaling pathway has recently been shown to be involved in the regulation of cell proliferation and migration in BCa (23,24). Therefore, we hypothesized that the overexpression of SDF-1 and CXCR4 may promote cell proliferation, migration and invasion in BCa through activation of the Wnt/β-catenin signaling pathway.

In the present study, we explored the association between the SDF-1/CXCR4 pathway and β-catenin and the regulatory roles of SDF-1/CXCR4 in the expression of β-catenin. Ultimately, our findings demonstrate a new signal transduction pathway, the SDF-1/CXCR4/β-catenin axis, which was activated and promoted proliferation, migration and invasion in BCa cells.

Materials and methods

Cell culture. Three BCa cell lines (SW780, 5637 and T24) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in Invitrogen™ RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing Gibco™ 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin at 37˚C in 5% CO₂.

SW780 cells (200, 400 and 800 cells/well, respectively) were placed in a fresh 6-well plate following 2 days, the cells were collected for RNA isolation, proliferation, colony formation, migration and invasion assays.

MTS assay. SW780 cell proliferation was monitored using the MTS assay kit (Promega Corp., Madison, WI, USA). Absorbance was assessed at 492 nm using an ELISA reader (MD SpectraMax M5; Molecular Devices, LLC, Sunnyvale, CA, USA). The detailed documentation for the MTS assay was performed as previously described (25).

Colony formation assay. SW780 cells (200, 400 and 800 cells/well, respectively) were placed in a fresh 6-well plate containing 10% FBS. To verify the association between the SDF-1/CXCR4 pathway and β-catenin, the cells were treated with PBS, AMD3465, SDF-1, FH535, AMD3465+SDF-1 or FH535+SDF-1 for 48 h; the concentrations of AMD3465, SDF-1 and FH535 were 10 µM, respectively, and were plated in a 6-well plate at a density of 2x10⁵/well. Following incubation for 2 days, the cells were collected for RNA isolation, proliferation, colony formation, migration and invasion assays.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using Invitrogen™ TRIzol (Thermo Fisher Scientific, Inc.). Moloney Murine Leukemia Virus Reverse Transcriptase (Promega Corp.) and Oligo(dT)₅ primers (Thermo Fisher Scientific, Inc.) were used to synthesize cDNA, which served as the template for the PCR performed using a DNA Engine (ABI 7300; Thermo Fisher Scientific, Inc.). The reaction mixtures (20 µl) were prepared using the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Inc.) and the reaction conditions were carried out according to the manufacturer's protocol. The PCR primers used were as follows: For CXCR4 forward, 5’-ATCAATCTG AGACGCTAACC-3’ and reverse, 5’-CACCATTTTGAGCCA ACAGC-3’; for β-catenin forward, 5’-GGCTCTGTAAT AGGCCTACTTGT-3’ and reverse, 5’-ACGCAAGGTGC ATGGTTT-3’; for β-actin forward, 5’-CATGTACGTTGC TATCCAGGC-3’ and reverse, 5’-TACCTTAATGTTCAG CACGAT-3’; β-actin served as a housekeeping gene. The relative expression levels of genes were calculated using the 2⁻ΔΔCq method (28).

Western blotting. Protein was extracted using RIPA lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China). The concentration was determined using the Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich; Merck KGaA). Samples containing 50 µg of protein were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primary antibodies p-β-catenin (dilution 1:1,000; cat. no. 55-1100; Cell Signaling Technology, Inc., Danvers, MA, USA), β-catenin (dilution 1:1,000; cat. no. sc7199; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), MMP-2 (cat. no. ab31750; dilution: 1:2,000; Abcam, Cambridge, UK), c-myc (dilution 1:1,000; cat. no. ab32072; Abcam), E-cadherin (dilution 1:1,000; cat. no. 14472; Cell Signaling Technology), N-cadherin (dilution 1:1,000; cat. no. sc53488; Santa Cruz Biotechnology, Inc.) were incubated with the membrane for 2 h at room temperature, and then the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (dilution, 1:10,000; cat. no. sc-516102; Santa Cruz Biotechnology, Inc.), following visualization using chemiluminescence reagent (Thermo Fisher Scientific, Inc.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; dilution...
1:2,000; cat. no. 2118; Cell Signaling Technology, Inc.) was used as the control antibody. Signals were densitometrically assessed using Quantity One® software version 4.5 (Bio-Rad Laboratories, Inc.).

Animal experiments. The present study was approved by the Ethics Committee of Beijing University of Chinese Medicine at Shenzhen Hospital (Shenzhen, China). A total of 10 BALB/c 6-8 week old, athymic nude mice (body weight, 25-30 g) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China) and acclimated to the environment for 1 week. Animals were maintained in a specific pathogen-free (SPF) environment throughout the experiments, with a controlled humidity (50±10%), light (12-h light/dark cycle), and temperature (23±2˚C), fed with food and water ad libitum. Establishment of the subcutaneous tumor model was performed as previously described (29). All of the mice received a subcutaneous injection of SW780 cells (1x10^6). Tumor growth was assessed every week using a dial caliper and tumor volume (V) was calculated by the following formula: V = πAB^2/6, in which A is the largest diameter and B is the perpendicular diameter. After the tumor volume reached 100-200 mm^3, the mice were divided into two groups receiving PBS (control group; n=5) or AMD3465 treatment (AMD3465 group; n=5). The mice in the two groups were injected with 500 µg AMD3465 or an equal volume of PBS subcutaneously respectively, once per day until they were sacrificed, with an intraperitoneal injection of sodium pentobarbital (200 mg/kg; Sigma-Aldrich; Merck KGaA) 12 weeks after inoculation. The tumor volume of the two groups was assessed twice every week until the time of sacrifice, and the tumors were isolated and weighed after the sacrifice.

Statistical analysis. The data from these experiments were reported as the mean ± standard deviation (SD) for each group. All statistical analyses were performed with PRISM version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). The Student’s t-test was used to analyze differences between two groups. Inter-group differences were analyzed by one-way analysis of variance (ANOVA), followed by a post hoc Tukey’s test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

CXCR4 and β-catenin mRNA expression in BCa cell lines. To select suitable cell lines for the study of the molecular mechanisms, we examined the mRNA levels of CXCR4 and β-catenin in three BCa cell lines (SW780, 5637 and T24) using RT-qPCR analysis. CXCR4 mRNA levels were the most highly expressed in the SW780 cell line (Fig. 1A). The mRNA levels of β-catenin were highly expressed in both the SW780 and 5637 cell lines (Fig. 1B). Therefore, we focused on the association between SDF-1/CXCR4 and β-catenin in the SW780 cell line.

Abrogation of CXCR4 with AMD3465 inhibits cell proliferation, colony formation, migration, invasion and β-catenin protein expression. The SW780 cells were exposed to AMD3465 (10 µM), a CXCR4 inhibitor, and then MTS and a colony formation assay were performed. We found that the growth (Fig. 2A) and colony formation ability (Fig. 2B) of AMD3465-treated SW780 cells were significantly suppressed compared to those of the control group. In order to examine the effect of AMD3465 on cell migration and invasion, SW780 cells were cultured on a Transwell apparatus and a Boyden chamber coated with Matrigel. Following a 12-h incubation, the migration (Fig. 2C) and invasion (Fig. 2D) capacities were also observed to be significantly inhibited in the AMD3465-treated group compared with the control. These data indicated that overexpression of CXCR4 may be oncogenic and promote metastasis, and that CXCR4 loss of function can inhibit a migratory and invasive phenotype in SW780 cells.

As displayed in Fig. 2E, suppression of CXCR4 expression decreased the protein expression of β-catenin and its targeting genes (MMP-2 and c-myc) involved in Wnt/β-catenin signaling-mediated cell proliferation, invasion and differentiation to enhance cancer progression (30). The expression of the epithelial cell marker E-cadherin was significantly increased in the AMD3465-treated SW780 cells compared with the control. However, the protein expression of p-β-catenin and mesenchymal cell marker N-cadherin showed no obvious differences between the two groups.
SDF-1 reverses the inhibitory effect of AMD3465 on cell proliferation, colony formation, migration and invasion in SW780 cells. In view of the competitive relationship between SDF-1 and AMD3465 to bind with CXCR4 (31), we hypothesized that the complete opposite effect of SDF-1 and AMD3465 would be observed on the progression of BCa. As expected, SW780 cell proliferation was significantly increased 72 h after SDF-1 treatment, compared with the control group (Fig. 3A). In addition, the numbers of cloned (Fig. 3B and C), migratory (Fig. 3D) and invasive (Fig. 3E) SW780 cells were significantly greater in the SDF-1 treatment group compared with the control. AMD3465 had a completely contrary effect.
Figure 3. SDF-1 reverses the inhibitory effect of AMD3465 on cell proliferation, colony formation, migration and invasion in BCa SW780 cells. (A) After the SW780 cells had been treated with PBS, SDF-1 (100 ng/ml), AMD3465 (10 µM), or AMD3465+ SDF-1 for 24, 48 and 72 h, the cell viability was assessed using an MTS assay. (B and C) Colony formation, (D) migration, and (E) invasion assays were performed after treatment with PBS, SDF-1, AMD3465, or AMD3465+ SDF-1 for 48 h. (F) The protein expression of p-β-catenin, β-catenin, MMP-2, c-myc, E-cadherin, and N-cadherin were determined by western blotting, after being treated with PBS, SDF-1, AMD3465, or AMD3465+ SDF-1 for 48 h. (G) Histograms are representative of the protein quantification. n=3 in each group. *P<0.05, **P<0.01 compared with the control group; $$P<0.01 compared with the SDF-1 treated group. BCa, bladder cancer.
on cell proliferation, colony formation, migration and invasion in SW780 cells (Fig. 3A-E). Notably, SDF-1 significantly reversed this inhibitory effect (Fig. 3A-E).

Furthermore, the effects of SDF-1 on the protein expression of p-β-catenin, β-catenin, MMP-2, c-myc, E-cadherin and N-cadherin in SW780 cells were evaluated. SDF-1 treatment had no significant effect on any of these factors, except for p-β-catenin (Fig. 3F and G). c-myc was significantly upregulated, and E-cadherin was downregulated in the group treated with SDF-1 combined with AMD3465 in SW780 cells.
compared with AMD3465 treatment alone. However, SDF-1 had no significant effect on p-β-catenin, β-catenin, MMP-2, or N-cadherin in AMD3465-treated SW780 cells (Fig. 3F and G). These findings indicated an inverse relationship between SDF-1 and AMD3465 on cell proliferation, colony formation, migration and invasion in SW780 cells.

SDF-1 reverses the inhibitory effect of FH535 on cell proliferation, colony formation, migration and invasion in SW780 cells. To investigate the role of β-catenin in the progression of BCa, we treated cells with a β-catenin antagonist, FH535. First, we found that the protein expression of p-β-catenin was significantly inhibited in a concentration-dependent manner by FH535 treatment of SW780 cells (Fig. 4A and B). In addition, the protein expression of β-catenin was not evidently different in SW780 cells with FH535 treatment (Fig. 4A and B). Compared with the control group, cell proliferation was significantly inhibited by FH535 treatment at 72 h (Fig. 4C). The down-regulation of β-catenin by FH535 treatment led to a reduction in the number of cloned (Fig. 4D), migratory (Fig. 4E), and invasive (Fig. 4F) SW780 cells. Interestingly, SDF-1 treatment significantly reversed the inhibitory effect of FH535 on cell proliferation, colony formation, migration and invasion in SW780 cells (Fig. 4C-F). These findings indicated that SDF-1 plays an important role in the regulation of the expression of β-catenin during the development of BCa.

Effect of AMD3465 on SW780 cell xenograft growth in the nude-mouse model. We performed a tumor xenograft assay and found that tumors from the control group grew faster than those of the AMD3465-treated group during the entire tumor-growth period (Fig. 5A). Twelve weeks after inoculation, the average weight of tumors from the AMD3465-treated group was markedly lower than those of the control group, indicating that AMD3465 inhibited SW780 cell growth in vivo (Fig. 5B and C).

Discussion

The majority of bladder cancer (BCa) patients have locally advanced or metastatic disease and the pelvic lymph node is the primary site of metastasis, followed by widespread metastasis to the lung, liver, bone marrow or other tissues (17). A previous study indicated that cancer metastasis is not an unordered process (32), as adhesion molecules (33), cytokines (34) and chemokines (16) may play a crucial role in the process of BCa metastasis. Among these key regulators, chemokines and their receptors may be the representative regulators (35).

In the present study, we highlighted the involvement of the SDF-1/CXCR4 signaling pathway in the progression of BCa. The in vitro results were consistent with the in vivo nude-mouse experiments which revealed that decreased CXCR4 expression by AMD3465 inhibited SW780 cell growth. In addition, migration and invasion of SW780 cells were both inhibited by a CXCR4 antagonist. These results were similar to those of previous studies (17,36) and also indicated that the high metastatic potential of human BCa cells in vitro was closely associated with increased CXCR4 expression.
We further revealed that a CXCR4 antagonist can inhibit β-catenin protein expression. Previous studies indicated that β-catenin may be a biomarker for the metastatic progression of BCa patients and that it was associated with poor patient survival (37,38). Wnt/β-catenin signaling has been shown to be frequently involved in various signaling pathways related to cell proliferation and metastasis in BCa (39,40). For example, hepatocyte cell adhesion molecule (hepCAM) specifically suppressed β-catenin expression during BCa cell proliferation (39). In addition, long non-coding RNA H19 increased BCa metastasis by activating the Wnt/β-catenin signaling pathway (40). Thus, our results showed that the CXCR4 antagonist induced the inhibition of cell proliferation, colony formation, migration and invasion, at least partially, by downregulating the expression of β-catenin.

β-catenin and its downstream target genes, c-myc and MMP-2, are associated with tumorigenesis and metastasis in multiple types of cancer (30,41). Numerous studies have shown that the suppression of the c-myc oncogene induced cellular senescence in BCa (42,43). MMP-2 is an enzyme that has been implicated in the malignant progression of BCa (44). Usually, β-catenin, c-myc, and MMP-2 have a synergistic effect on BCa tumorigenesis and progression (39,45). In the present study, the suppression of CXCR4 expression also decreased the protein expression of β-catenin targeting genes (MMP-2 and c-myc). Our results confirmed the previously established role of the CXCR4/β-catenin axis in the malignant progression of cancers (18-20).

When investigating the interaction of chemokines and chemokine receptors, SDF-1 was proven to be a stimulator of migration and invasion in CXCR4-positive cancer cells (46), suggesting that SDF-1 may play a central role in the SDF-1/CXCR4 axis. Previous studies revealed that the addition of SDF-1 significantly induced the proliferation, migration and invasion of colon cancer cells (20,47). Song et al also found that β-catenin was recruited in the nuclei to stimulate cell proliferation in the presence of SDF-1, but SDF-1 had no obvious effect on β-catenin in a whole cell lysate (19). In this study, SDF-1 was revealed to induce the phosphorylation of β-catenin, which has been shown to promote β-catenin escape, disruption of E-cadherin association and the disassembly of adherent junctions, which all lead to cancer cell proliferation (48). Concurrently, SDF-1 could reverse the inhibitory effect of the β-catenin antagonist on cell proliferation, colony formation, migration and invasion in SW780 cells. All of these findings indicated that β-catenin was a promising key target gene in the SDF-1/CXCR4 axis in BCa metastasis.

To the best of our knowledge, this is the first study that revealed the association between SDF-1/CXCR4 signaling and the activation of β-catenin in the malignant progression of BCa. Nevertheless, we also found that SDF-1/CXCR4 may induce epithelial-mesenchymal transition (EMT) by inhibiting the E-cadherin expression involved in BCa metastasis; however, further investigation of this mechanism is required.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

TZ conceived and designed the study, drafted and revised the manuscript. FY performed the experiments, analyzed the data and drafted the manuscript. WenbL, ZC, CW, BL and WendL helped in study design, study implementation and manuscript revision. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Beijing University of Chinese Medicine at Shenzhen Hospital and approved the animal research carried out.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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